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(72) Inventors JAN BOLDINGH and CORNELIS OKKERSE

(54) PREPARATION OF ENZYMATICALLY ACTIVE SUBSTANCES

(71) We, UNILEVER LIMITED, a company registered under the laws of Great Britain, of Port Sunlight, Wirral, Cheshire, England, do hereby declare the invention, for which we pray that a patent may be granted to us, and the method by which it is to be performed, to be particularly described in and by the following statement:—

This invention relates to enzymatically active preparations, a method for their preparation and to processes for the use of these substances for carrying out enzymatic reactions.

It is known to carry out chemical reactions in, for example, aqueous media *in vitro* by means of enzymes. However, the separation of the reaction products from the enzyme is often difficult and the enzyme can only be used once, because it mostly loses its activity during the separation procedure. It has therefore been suggested to fix enzymes to a carrier which is insoluble in the medium in which the enzymatic reaction is to be carried out. The insoluble carrier-enzyme material can be used to carry out enzymatic reactions batchwise in which the carrier-enzyme material and substrate are mixed and the resulting mixture is usually agitated. It is also possible to carry out these enzymatic reactions continuously by percolating a substrate solution through a bed of carrier-enzyme material. In these cases the separation of the enzyme from the reaction mixture does not present difficulties.

It has been suggested to incorporate enzymes in high molecular weight organic carrier material such as, for example, cellulose derivatives. The carrier material is dissolved in an organic solvent or a mixture of solvents to which a dry enzyme preparation is added, after which the solvent is removed and the high molecular weight material, in which enzymes are embedded, is obtained. It has further been suggested to prepare an enzymatically active synthetic resin material, such as a polyacrylamide resin, by polymerising acrylamide in a medium containing a cross-linking agent and a polymerisation catalyst in the presence of an enzyme.

Both methods involve the use of organic reagents which can affect the activity or stability of the enzyme. These methods also have the disadvantage that only a small part of the active enzyme is really embedded in such a way that it cannot be washed out.

According to one aspect of the present invention, there is provided an enzymatically active preparation comprising a silica hydrogel containing at least 10% water by weight of the silica in which an enzyme is entrapped.

The invention also relates to a method for preparing such enzymatically active preparations in which a silica hydrogel is prepared in the presence of an enzyme.

A silica hydrogel can be obtained by gelation of a silica sol prepared, for example, by the addition of an aqueous solution of an alkali metal silicate to a mineral acid under carefully controlled conditions of temperature and pH. Primary particles of a polysilicic acid are formed in this sol which interlink during subsequent gelation of the sol to form a network containing a series of pores, the size of which is dependent on the size of the primary particles. The primary particles will vary in size from a few to several hundreds of nanometers (1 nano metere = 10^{-9} metre) in diameter. The silica hydrogel usually contains an amount of water varying from 10—5,000%, preferably 10—500%, by weight of its silica content. The amount of water present in an enzymatically active preparation based on a silica hydrogel is dependent on the nature of the enzyme entrapped. The silica hydrogel will vary in appearance from a jelly-like material to a hard granular material according to the amount of water it contains. The term "silica hydrogel", as referred to in this specification, does not apply to a silica gel of water content less than 10% by weight SiO_2. Silica xerogel is an example of a silica gel of this type.

The enzyme used in the process of this

invention may be any enzyme or combination of enzymes in the dissolved, dispersed, suspended, emulsified or dry state. Enzymes contaminated with proteolytic enzymes which otherwise could break down the enzyme can be entrapped with success by the process of this invention and maintain their activity. Examples of enzymes which can be used to form an enzymatically active preparation include acetyl choline esterase, chymotrypsin, trypsin, alkaline phosphatase, catalyse, lipoxidase and prostaglandin synthesizing enzyme.

The process according to the invention is also particularly suitable for incorporating enzymes which occur in particle bound enzymes, cell organellae, cell fragments, lyophilized or dehydrated cells.

The conditions employed during the formation of the enzymatically active preparation can vary widely being primarily dependent on the stability of the enzyme toward chemicals and heat. Thus, with some enzymes it may be necessary to observe special precautions in order to retain their activity such as carrying out the formation of the enzymatically active preparation in an inert gas atmosphere and/or at a low temperature.

Although preferred, it is not absolutely necessary when preparing the silica hydrogel-enzyme system to add the enzyme before the silical sol is gelled. The addition may also be effected during the initial stages of the gelation. In order to avoid decay of the enzyme by micro-organisms, preserving agents, such as tetracyclines, can be added at an appropriate stage of preparation or use.

Providing that the conditions such as pH or temperature are suitable for the entrapped enzyme, the silica hydrogel-enzyme preparation, which usually has a jelly-like appearance, can be solidified by dehydration. The dehydrated product can be given various forms by, for example, cutting and subsequently producing lamellae by drying, by coating, brushing or spraying other objects before drying, by spray-drying and by granulation before completion of drying. It is also possible to carry out the gelation of the silica sol containing the enzyme, in the presence of a suitable porous carrier such as pumice, porous plastic or porous glass beads so that the silica hydrogel-enzyme preparation is deposited on or in the carrier. Gelation of aqueous silica sol can take place spontaneously but generally it is desirable to accelerate gelation, which may be done by, for example, addition of small amounts of electrolytes (salts) and adjustment of pH in accordance with the enzyme to be entrapped.

Dehydration of the silica hydrogel-enzyme system should not be continued beyond the water content at which the enzymatic activity drops sharply. At a water content of 10% calculated on the silica content of the aqueous silica sol the enzymatic activity of most enzymes which can be embedded in the silica gel has practically disappeared.

It has been found that enzymatic processes can be effected by passing a solution of a substrate through columns of, or columns containing, the silica hydrogel-enzyme preparation optionally in admixture with suitable inert volume extenders, the rate and degree of conversion being a function of the parameters of the column and of the rate of flow through the column. The use of columns containing enzymatically active material has the advantage that due to the flow principle the reactant concentration can be maintained optimum. The use of silica hydrogel-enzyme preparations which are prepared by the process of this invention and are deposited on or in porous material in columns is particularly advantageous with respect to flow rate and efficiency of conversion.

Multiple step conversions of substrate can be effected by using a combination of enzymes entrapped in the silica hydrogel or by a combination of separate silica hydrogel-enzyme preparations either in one column or in several beds or columns in sequence. The silica hydrogel-enzyme preparations according to the invention show a good stability on storage and can be used for synthetic and analytical purposes.

The silica hydrogel-enzyme systems according to the invention and their preparation and use show the following interesting features:—

1. The enzymes can be incorporated in, for instance, the dissolved or dispersed state, thus eliminating the necessity of converting them into the dry form before use which could entail loss of enzymatic activity.

2. The silica hydrogel-enzyme system, though hydrated, can easily be handled as a solid or semi-solid substance, whereas the microenvironment of the entrapped enzymes closely resembles water, which is the natural medium of enzymes where they usually display their highest activity.

3. The silica hydrogel-enzyme systems are prepared in the absence of organic reagents and using mild conditions like ambient temperature, addition of minor quantities of electrolytes (salts), pH conditions of usually from 5—8, whereby gelation of the silica sol can be brought about within a period varying from some minutes to several hours.

4. With silica hydrogel the amount of incorporated water can be adjusted within wide ranges from 10 to 5,000% of water based on the weight of the silica content to

meet the requirements of the enzyme and substrate.

5. Silica hydrogel has an "open" structure and is easily accessible to substrate molecules of a wide range of molecular size.

6. The silica hydrogel network consists of interlinked spherical primary particles which can vary from about a few to several hundred nanometers. The sizes of the pores vary with the size of the primary particles and may therefore be adjusted so as to obtain a preparation of optimum activity.

A typical preparation of a silica sol suitable for use in the process of the invention will now be described.

100 ml triple-distilled water were put into a vessel which was equipped with the electrodes of an automatic titration unit. With vigorous stirring, 520 ml 2N hydrochloric acid was added to the distilled water in a constant stream. A solution obtained by mixing 260 ml sodium silicate solution (molar ratio $Na_2O:SiO_2 = 1:3.42$), having a gravity of 41°Be, with 240 ml triple-distilled water was dosed from an automatic titration unit, in such a way that the pH in the reaction vessel was kept at a constant value of 1.6. The temperature was maintained at room temperature.

The clear silica sol obtained, containing 8.2% by weight silica and about 4.7% by weight sodium chloride was dialysed in a Cellophane dialysing tube of 28 mm diameter against a constant stream of dilute hydrochloric acid of pH 1.6 for about 20 hours. (The word "Cellophane" is a Trade Mark). Per charge of 1.3 l silica sol, a total of 100—120 l dilute hydrochloric acid was applied. After dialysis, the sol contained 5.5—6.5% by weight silica and about 0.02% by weight sodium chloride. On storing the sol at 0°C, no gelatinous precipitate was formed until 4—5 weeks had lapsed.

The invention will now be described with reference to the following Examples:—

EXAMPLE 1

3.8 mg of an acetyl choline esterase preparation were dissolved in 1 ml of water. 25 mg potassium dihydrogen phosphate were dissolved in 25 ml of a freshly prepared silica sol (prepared by the method as described above). The pH of this silica sol mixture was adjusted to 7.5. The dissolved enzyme was then quickly added to 9.4 g of the pH adjusted silica sol, after which the mixture gelled. The silica hydrogel-enzyme preparation thus obtained contained per gramme, 0.365 mg of the acetyl choline esterase preparation. The enzymatic activity of the silica hydrogel-enzyme preparation was determined as follows:

2 g of the silica hydrogel-enzyme preparation were pressed through a sieve (mesh width 0.1 mm) and added to 11 ml of a 0.01 molar phosphate buffer (pH 7.4) in a vessel equipped with the electrodes of an automatic titration unit. 3 ml of an aqueous 0.2 molar magnesium chloride solution were added. The pH of the mixture was adjusted to 7.4 until constant. This had to be done several times because of the exchange capacity of the silica hydrogel. Subsequently 0.5 ml of an aqueous 0.055 molar solution of acetyl choline hydrochloride were added (which is converted by the acetyl choline esterase to acetic acid and choline hydrochloride). The acetic acid formed was titrated by means of an automatic titration unit with 0.02 N aqueous sodium hydroxide and the activity of the acetyl choline esterase was determined as sodium hydroxide consumption expressed in g equivalents per min. By comparing the activity thus determined with the activity of 1 mg of the starting enzyme material in the dissolved state, the relative activity of the embedded enzyme material was determined. A relative activity of 62% was found.

In order to investigate whether absorption or entrapment of the enzyme was involved, the silica hydrogel-enzyme system was rinsed with a 10% aqueous solution of methylamine hydrochloride (which can be used to desorb absorbed proteins) and subsequently three times with portions of the 0.01 molar phosphate buffer. The silica hydrogel-enzyme preparation showed a relative activity of 58% when compared with the starting enzyme material.

A quantity of 1.6 g silica hydrogel was stirred for one and a half hours in a solution comprising 2 mg of the starting acetyl choline esterase preparation in 4 ml of water. After discontinuing stirring the mixture was centrifuged. The supernatant liquid contained 1.48 mg active enzyme material. The silica hydrogel was rinsed three times with quantities of the 0.01 molar phosphate buffer. The silica hydrogel then showed an enzymatic activity corresponding to 0.05 mg of the enzyme.

The enzyme entrapped in silica hydrogel as prepared according to this experiment was compared with the same enzyme embedded in a polyacrylamide gel prepared from the following ingredients:—

Solution A:
40 g of acrylamide in 100 ml N/10 phosphate buffer, pH 7.4.

Solution B:
2.3 g of N,N¹-methylene bis(acrylamide) in 100 ml phosphate buffer, pH 7.4.

Solution C:
3 mg riboflavin in 10 ml N/10 phosphate buffer, pH 7.4.

Solution D:
3 mg ammonium persulphate in 10 ml N/10 phosphate buffer, pH 7.4.

Solution E:
6 mg acetyl choline esterase in 1 ml phosphase buffer, pH 7.4.

The polyacrylamide/gel-enzyme preparation was made by mixing 1 ml of solution A with 4 ml of solution B and adding 1 ml of the enzyme solution E. The photo polymerization by direct sunlight was catalysed by adding 0.1 ml of solution C and 0.1 ml of solution D. The polymerisation was completed in about 10 min. The gel was finely dispersed by pressing it through a sieve (0.1 mm). Although this preparation showed a relative activity of about 66%, this activity readily diminished upon rinsing to about 5%, the rinsing fluid thus contained about 63% of the enzymatic activity.

A comparison of acetyl choline esterase entrapped in silica hydrogel with the same enzyme embedded in a cellulose nitrate membrane was also made as follows.

10 mg of dry acetyl choline esterase were suspended in 10 ml of a solution containing by weight 2.1% celiulose nitrate, 52.8% diethylether, 14.1% ethyl alcohol, 20.9% acetone, 6.4% amyl alcohol and 3.7% ethyleneglycol monoethyl ether. The suspension was poured onto a flat glass dish with a surface area of 44 cm^2 and for 75 minutes the volatile components were evaporated by a gentle nitrogen flow. The membrane thus formed, was flooded with 100 ml water and cut into little pieces. The 100 ml water contained the greater part of the enzyme activity, the membrane only about 7%, which further diminished upon rinsing to less than 0.5%.

Example 1A

In the same way as described in Example 1 a quantity of 6 mg of an acetyl choline esterase preparation was entrapped in 50 ml silica hydrogel. The silica hydrogel-enzyme preparation obtained was dehydrated to a water content of about 360% by weight of the silica content, based on SiO_2. The dehydrated gel was carefully pulverised in a mortar and sieved. From the sieve fraction, having dimensions of between 0.6 and 0.25 mm, the fines were removed by washing and decantation. A column with an inner diameter of 7 mm was filled with 3.5 g of these particles, equivalent to at most, 1.77 mg of the acetyl choline esterase preparation. This column was kept at 25°C with the aid of a thermostat. A substrate solution consisting of 0.33 mg/ml acetyl choline hydrochloride in 0.01 molar phosphate buffer solution (pH 7.4) and additionally containing 8 mg/ml $MgCl_2.6H_2O$ and 100 mg/l oxytetracycline and 1 ml/l pimafucine suspension (2.5%) was led through the column.

During the experiment the stock solution was held just above 0°C and only warmed up when entering the column.

At a rate of 75 ml/h 80% of the acetyl choline hydrochloride was converted. By lowering the rate of flow to 37.5 ml/h all the substrate was converted. This column was constantly used for four days, during which time no loss of activity was observed.

Example 1B

In the same way as described in Example 1, 6.8 mg acetyl choline esterase were added to 9 ml silica sol (adjusted to pH 7.4) and this mixture was added to 3.12 g pumice (particle diameter about 3 mm) in an evacuated vessel. Atmospheric pressure was subsequently restored in order to fill all the pores of the pumice with the enzyme-sol mixture. That part of the sol which was not retained by the pores was removed by sucking over a glass filter.

2.48 g sol (containing 1.69 mg enzyme) was retained by the pumice. After gelling, the dehydrated particles were put in a column which was used as described in Example 1A. At a pumping rate of 18 ml/h 85% of the substrate was converted. This activity was constant during the time of the experiment, being 5 days.

Example 2

200 mg KH_2PO_4 were dissolved in 100 ml dialysed silica sol, prepared according to the method described above. The pH was adjusted to 3—4 by the addition of a 4N NaOH solution. 30 mg trypsin were dissolved in 5 ml 10^{-2}N aqueous hydrochloric acid and added to the sol. The pH of the resulting mixture was adjusted to 8.0 by further adding 4N NaOH. After several minutes a firm, solid hydrogel (SiO_2 content 5.2—6.2%) had formed.

The trypsin activity of this hydrogel-enzyme system was determined as follows. 2 g of the hydrogel were pressed through a sieve having meshes of 0.1 mm diameter and subsequently added to 15 ml of a 0.01 molar phosphate buffer that had been adjusted to a constant pH of 8.0. Subsequently 100 mg N-alpha-benzoyl-arginine ethyl ester (BAEE), dissolved in 0.5 ml of the aforementioned phosphate buffer, were added. The activity of the trypsin was measured in a water thermostat by continuously titrating the acid with a 0.0025 N NaOH solution using an automatic titration unit set at pH 8.0 (the temperature being kept constant at 25°C). The NaOH consumption per minute provided a measure for the activity of the enzyme, which is expressed in gram equivalent per minute per gram of enzyme. Because of the ion-exchange activity of the silica hydrogel, the pH was disturbed again after the addition of the BAEE and the rate of consumption of NaOH had to be determined

after a certain quantity of NaOH had already been added. This quantity of NaOH was determined in the same way in a blank experiment, carried out with an equal amount of silica hydrogel in the absence of enzyme. The activity of the preparation was compared with the same amount of dissolved enzyme as was entrapped in the silica hydrogel. Thus a relative activity of 55—65% was found.

The hydrogel-enzyme system was dehydrated under vacuum over concentrated sulphuric acid until its water content was about 200% by weight of the silica present. It appeared that no enzyme activity was lost during this dehydration step. Also after rinsing the granules several times with either triple-distilled water or the above-mentioned buffer solution it appeared that the activity had not diminished.

A comparison of the stability at 25°C of dissolved trypsin with that of trypsin entrapped in silica hydrogel was made as follows. A stock solution of trypsin in 10^{-3}N HCl containing 1 mg enzyme material per ml was prepared. This solution is stable for several hours and was used in the following experiment:

0.2 ml trypsin solution were added to a solution of 100 mg BAEE in 15 ml (0.01 molar) phosphate buffer at pH 8.0. It was found that 5.7×10^{-4} gram equivalent BAEE were converted per minute.

In the subsequent four experiments the procedure was repeated with the 0.2 ml trypsin solution being added to the buffer. After varying time intervals of 3, 15, 45 and 60 minutes respectively, 100 mg of BAEE were added and the rate of conversion was determined. The conversion rates found are tabulated below:

Period of enzymatic reaction after t=0 (min)	Conversion rate g.eq./min.
0	5.7×10^{-4}
3	4.5×10^{-4}
15	4.0×10^{-4}
45	3.5×10^{-4}
60	3.2×10^{-4}

Furthermore, the conversion rate of a 150 mg quantity of the partly dehydrated silica hydrogel containing entrapped trypsin was tested after 15 ml of phosphate buffer had been added (cf. the activity determination described above). A conversion rate of BAEE of 5.1×10^{-4} g.eq./min. was found. The silica hydrogel-enzyme system was filtered off and in a subsequent similar experiment a further quantity of BAEE was converted. The activity found was 4.9×10^{-4} g.eq./min., which remained almost constant in the following conversions. The procedure was repeated after allowing the original hydrogel-trypsin system to stand for 1 and 2 hours and the same conversion rates were observed.

In a comparative Examyle 6 mg trypsin were included in polyacrylamide gel in the way described in Example 1. Although this preparation showed a relative activity to about 74%, this activity decreasecd upon rinsing to about 7%, the rinsing fluid containing about 68% of the activity.

Example 3

In the same way as described in Example 2 a quantity of 300 mg trypsin was embedded in silica hydrogel instead of the 30 mg mentioned in Example 2. The silica hydrogel-enzyme showed about 70% of the activity of the trypsin used in preparing the hydrogel-enzyme system. The gel thus obtained could also be dehydrated without further loss in activity until a water content equal to 300% by weight of the SiO_2 had remained.

Example 4

As described in Example 2, a silica hydrogel-trypsin preparation was prepared from only 2 mg trypsin instead of 30 mg mentioned in Example 2 and dehydrated until a water content equal to about 300% by weight of the SiO_2 remained. The dehydrated gel was carefully pulverised in a mortar and sieved. 3 g of the particles having dimensions of between 0.6 and 0.25 mm, and containing at most 0.33 mg trypsin were added to a column with an inner diameter of 7 mm until it was filled to a bed height of 7 cm. This column was kept at 25°C with the aid of a thermostat. A solution of 1 mg/ml N-alpha-benzoyl-aginine ethyl ester (BAEE) in a 0.01 molar phosphate buffer (pH 8) in which 400 mg SiO_2 per litre had been dissolved was led through the column at a rate of 1.2 ml/min. The BAEE solution led through the column was completely converted in the time of the experiment, which was 72 hours. In this period altogether about 5 g BAEE were converted.

Example 5

30 mg $MgSO_4$ (anhydrous) were dissolved in 25 ml dialysed silica sol, obtained as described above. This solution was adjusted to pH 7 with 4N NaOH solution, after which 30 mg of alkaline phosphatase dissolved in 6 ml of a "tris" buffer solution, were added. This "tris" buffer was prepared by dissolving in 1 l water 0.01 mole tri-(hydroxymethyl) aminomethane and 0.01 mole $MgSO_4$ (anhydrous) and adjusting the pH to 8.8 with acetic acid. The mixture gelled and after half an hour the stiff mass was crushed with the aid of a spatula and thoroughly rinsed with further "tris" buffer solution. The rinsed mass weighed 29.3 g and thus con-

tained theoretically 1.2 mg enzyme per g of gel. The rinsed gel was pressed through a sieve with a mesh width of 0.1 mm.

A 0.01 molar solution of disodium phenyl phosphate in the above-mentioned "tris" buffer solution was prepared. 5 ml of this solution and 2 g of the rinsed and crushed particles of hydrogel-enzyme preparation were introduced into a tube provided with a ground glass stopper. The tube with its contents was continuously slowly turned over in order to effect a good mixing of the contents. After exactly 10 minutes, 2 ml of a 0.4 N NaOH solution were added to stop the reaction and the silica hydrogel was removed by centrifugation. The amount of phenol formed was determined by measuring the U.V. absorption at 278 nanometers in a 1 cm cell. By applying the same analytical procedure to various known quantities of dissolved alkaline phosphatase, the active enzyme content of the silica gel particles could be determined; this corresponded to 60% of the amout of enzyme which should theoretically be present in the sample.

Example 6

A silica hydrogel-lipoxidase preparation was prepared by a procedure similar to that described in Example 2. 7 mg lipoxidase were dissolved in 5 ml buffer (0.05 molar solution of NH_4Cl and NH_4OH of pH 9). The activity of the silica hydrogel-lipoxidase preparation so obtained was determined as follows.

The hydrogel was pressed through a sieve (size of mesh 0.1 mm). A substrate solution was prepared from 198 mg pure linoleic acid, which under gradual admixture of 2.05 ml 1N NH_4OH was rubbed into a homogeneous paste and emulsified in 98 ml water. 178 mg NH_4Cl were added to the emulsion which was saturated with oxygen. 2 g of the sieved gel were put in a tube with a ground glass stopper. 5 ml substrate were added at t=0 to the gel, after which the tube was continuously shaken. After exactly five minutes the tube was shaken with 5 ml butanol, after which its contents were centrifuged to obtain a quick separation into two phases. Of the upper phase (butanol phase) 2 ml were pipetted off and diluted with 6 ml methanol. Of this solution the U.V. absorption at 234 nanometers was measured, which absorption is a measure for the concentration of the conjugated diene system formed. By applying the same analytical procedure to various known standard solutions of lipoxidase in a 0.05 molar NH_4Cl-NH_4OH buffer of pH 9.0 and plotting the amount of lipoxidase against the extinction at 234 nanometers, it was possible, by graphical interpolation of the value found for the lipoxidase entrapped in the silica hydrogel, to determine the percentage of activity retained by the enzyme. Values of 50—60% of the original activity were found.

Example 7

A prostaglandin synthesising enzyme preparation (PGSE) was prepared in the following way.

25 g fresh sheep vesicular glands (connective tissue and fat being removed) were homogenised at 0°C, first in a household mixer and subsequently in a homogeniser in 50 ml 0.1 molar phosphate buffer (pH 7.5). This homogenate was centrifuged at 10.000 g for 15 minutes at 2—3°C. The supernatant was decanted and centrifuged for 1 hour at 100,000 g. This second centrifugate, which contained the PGSE, was freeze-dried. The yield was about 2 g consisting for about 30% of protein. Of this preparation, which should be considered as a particle bound enzyme preparation, 100 mg were emulsified in 5 ml 0.1 molar phosphate buffer (pH 7.5). 20 ml of silica sol prepared as described above were cooled to 0°C and the pH was adjusted to pH 6 with the aid of NaOH. The enzyme emulsion was then quickly added to this silica sol.

After this addition, the pH of the mixture was adjusted to a value of 7.5 when the mixture gelled.

Of the gel preparation thus obtained, 2 g (corresponding to 2 mg protein) were added to 2 ml phosphate buffer (pH = 7.5, 0.1 molar) to which 0.6 mg of the co-factor glutathion (Gamma-L-glutamyl-L-cysteinyl-glycine), 0.1 mg of the substrate dihomo-gamma-linolenic acid (all cis 8, 11, 14-eicosatrienoic acid) and 0.1 mg hydroquinone had been added. This mixture of silica hydrogel-enzyme preparation and the solution saturated with air and containing the substrate was shaken for 15 minutes at 35°C after which the reaction was stopped by acidification with citric acid to a pH valte below 3. Subsequently the mixture was shaken three times with 1 ml portions of diethyl ether. The ether layers were collected, combined and washed with water till the ether solution reacted neutral. Subsequently the ether solution was evaporated and the residue was taken up in 1 ml methanol. 0.1 ml of this solution was put in a 1 cm quartz cell together with 2.4 ml methanol and 0.5 ml 3N KOH in aqueous methanol (containing 75% methanol). After about 5 minutes the quantity of prostaglandin E_1 formed was determined from the increase of the U.V. absorption at 237 nanometers due to the dehydration and internal rearrangement of PGE_1. As compared with 2 mg protein of the same PGSE preparation in 2 ml buffer, stored at 0°C, the enzyme preparation entrapped in silica gel had retained 50% of its activity.

EXAMPLE 8

In a similar way to that described in Example 1 a quantity of urease was entrapped in silica hydrogel (pH 8). After gelation the hydrogel-enzyme preparation was dehydrated to a water content equal to about 150% by weight of the SiO_2. The resulting product contained 41 mg urease per g.

After pulverising and sieving the sample to particles smaller than 0.1 mm, the enzymatic activity of the silica hydrogel-enzyme preparation was determined by titration, in a way analogous to that described in Example 1. The buffer used was 0.1 molar phosphate buffer (pH 8.0) and the substrate was 300 mg urea in 3 ml buffer. The ammonia formed was titrated at constant pH with 0.2 N HCl. Though at pH 8.0 only about one half of the ammonia formed is titrated, which is the consequence of carbon dioxide also being formed, the activity of the urease preparation could be expressed in this way in g equivalents per min, which evidently is no absolute measure. By comparing the activity thus determined with the activity of 1 mg of the starting enzyme material in the dissolved state, the relative activity of the embedded enzyme was determined. A relative activity of about 40% was found. The activity was not affected by thoroughly rinsing the gel enzyme particles several times with the buffer used.

EXAMPLE 9

In a similar way to that described in Example 4, a column was made of 4.2 g hydrogel-urease particles, which contained per g about 20 mg urease preparation. Thus the column contained about 84 mg urease. A solution of 10 mg/ml urea in 0.1 molar phosphate buffer (pH 8.0), saturated with SiO_2 in a way similar to that described in Example 4, was led through the column. At a rate of 150 ml/h about 77% of the urea was converted; at a rate of 75 ml/h, 100% was converted.

EXAMPLE 10

A catalase preparation was entrapped in silica hydrogel in a way similar to that described in Example 1.

The preparation obtained contained per g about 1 mg of the enzyme. The hydrogel enzyme preparation was crushed and thoroughly rinsed.

The enzymatic activity of the silica hydrogel-enzyme preparation was determined as follows.

A suitable quantity of particles smaller than 0.1 mm was added to about 10 ml deaerated 0.1 molar phosphate buffer (pH 7.0). 0.2 ml of 0.3% H_2O_2 were added and the decrease in H_2O_2 concentration was measured in a known way with the aid of a platina calomel electrode system, while continuously removing the generated O_2 by bubbling N_2 through the reaction medium.

The H_2O_2 concentration was recorded logarithmically and a straight line was obtained. The slope of this line, expressed as sec^{-1}, was used as a measure for the activity of the preparations. By comparing the activity with the activity of 1 mg of the starting enzyme material, a relative activity of about 20% was found, which remained constant also after further thorough rinsing.

The enzymatically active preparations are also effective for the continuous production or both production of certain food flavours. The following two examples describe the preparation of a cheese flavour wherein *Penicillium roquefortii* spores and the wet cell material from *Cladosporium butyrii* are entrapped in a silica hydrogen and used to convert a substrate of fatty acids to methyl ketones.

EXAMPLE 11

A quantity of 1.8 g wet spores *Penicillium roquefortii* was suspended in a physiological solution to a total volume of 7 ml.

1 ml of this suspension containing about 10^{10} spores was added to 4.5 ml silica sol (adjusted to pH 7.0), after which the mixture was allowed to gel. The silica hydrogel thus obtained was crushed with a spatula and rinsed three times with 10 ml buffer solution (0.01 molar phosphate of pH 7). The rinsed particles were put in a 100 ml glass-stoppered bottle. A quantity of 20 ml of a substrate solution was added consisting of a 1 g/l solution of fatty acids in 0.01 M aqueous phosphate buffer (pH 7); the fatty acids consisted of 19.0% w/w acetic acid, 18.8% w/w butyric acid, 11.2% w/w caproic acid, 10.6% w/w caprylic acid, 19.6% w/w capric acid and 21.4% w/w lauric acid.

According to literature this is a good average composition of the C_2—C_{12} free fatty acids occurring in Scandinavian Blue cheeses and Roquefort cheese, 1 gram fatty acids being representative for about 250 g cheese.

Under aseptic conditions, the mixture of the fatty acids/buffer solution and the silica hydrogel-enzyme preparation was gently stirred at ambient temperature. After 18 hours the strong odour of methyl ketones was noticed, the total flavour perception closely resembling the characteristic aroma of Roquefort cheese.

The spore containing particles were removed by centrifuging, the supernatant fluid was analysed for methyl ketones. The following amounts were found (by means of gas chromatography), the figures between brackets showing the percentage of corresponding fatty acids converted:

2-heptanone 5.25 mg/l (4.8% on caprylic acid)
2-nonanone 6.50 mg/l (3.2% on capric acid)
2-undecanone 0.35 mg/l (0.2% on lauric acid)

No spores were found in the supernatant and the spores included in the silica gel particles did not germinate within the time of the experiment.

In a corresponding experiment using the same quantity of spores but without silica gel, the following amounts of methyl ketones were found:

2-heptanone 8.45 mg/l (7.7% on caprylic acid)
2-nonanone 4.95 mg/l (2.5% on capric acid)
2-undecanone 0.35 mg/l (0.2% on lauric acid)

It was further found possible to prepare these cheese flavours continuously using a column of the in silica embedded spores through which the substrate solution was percolated.

EXAMPLE 12

A quantity of 5 g wet cell material of *Cladosporium butyrii* was suspended in a physiological salt solution, the total volume being 10 ml containing about 9×10^{10} cells per ml. In a similar way to that described in Example 11, 1 ml of this suspension was included in a silica hydrogel, which was then crushed and rinsed, and mixed with 20 ml of the same substrate as used in Example 11.

After 20 minutes gentle stirring of this mixture, a cheese flavour was clearly percepted. After 18 hours the gel particles were removed by centrifuging and the methyl ketones formed were determined.

The following amounts were found (by gas chromatography):

2-heptanone ... 30.5 mg/l
2-nonanone ... 9.3 mg/l
2-undecanone ... 0.1 mg/l

No cells were found in the supernatant liquid and the cells included in the silica hydrogel particles did not germinate within the time of the experiment.

In a corresponding experiment using the same quantity of cells but without the silica gel, the following amounts were found:

2-heptanone ... 31.0 mg/l
2-nonanone ... 17.5 mg/l
2-undecanone ... 1.1 mg/l

WHAT WE CLAIM IS:—

1. An enzymatically active preparation comprising a silica hydrogel containing at least 10% water by weight of the silica in which an enzyme is entrapped.

2. An enzymatically active preparation as claimed in claim 1 which comprises an amount of water equal to 10—5,000% by weight of the silica content of the silica hydrogel.

3. A process for preparing an enzymatically active preparation as claimed in claim 1 or claim 2 in which a silica hydrogel is prepared in the presence of an enzyme.

4. A process as claimed in claim 3 in which the silica hydrogel is prepared by gellation of an aqueous silica sol.

5. A process as claimed in claim 3 or claim 4 in which the silica hydrogel is prepared in the presence of a dispersion or suspension of enzymes.

6. A process as claimed in claim 3 or claim 4 in which the silica hydrogel is prepared in the presence of a solution of enzymes.

7. A process as claimed in any one of claims 3—6 in which the silica hydrogel is prepared in the presence of a porous carrier.

8. A process as claimed in any one of claims 3—7 in which the silica hydrogel is dehydrated to water content of not less than 10% based on the weight of silica.

9. A process for the preparation of an enzymatically active preparation as claimed in claim 1 or claim 2 in which the enzyme occurs in particle bound enzymes, cell organellae, cell fragments, lyophilised or dehydrated cells.

10. An enzymatically reactive preparation as claimed in claim 1 or claim 2 that is prepared by a process as claimed in any one of claims 3—9.

11. A process for carrying out an enzymatic reaction which comprises mixing an enzymatically active preparation as claimed in claim 1, claim 2 or claim 10 with a solution of a substrate to be enzymatically converted, agitating the mixture for a predetermined duration and separating the enzymatically active preparation from the solution.

12. A process for carrying out an enzymatic reaction which comprises passing a solution of a suitable substrate through a column of an enzymatically active preparation as claimed in claim 1, claim 2 or claim 10.

13. A process for carrying out an enzymatic reaction as claimed in claim 11 or claim 12 substantially as herein described with reference to Examples 1—12.

14. An enzymatically converted reaction product whenever obtained by a method as claimed in any one of claims 11—13.

R. H. DOUCY,
Chartered Patent Agent.

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